Studying Werner syndrome to elucidate mechanisms and therapeutics of human aging and age-related diseases

Sofie Lautrup^{1,6}, Domenica Caponio^{1,2,6}, Hoi-Hung Cheung³, Claudia Piccoli^{2,4}, Tinna Stevnsner⁵, Wai-Yee Chan³, Evandro F. Fang^{1,*}

¹ Department of Clinical Molecular Biology, University of Oslo and Akershus University Hospital, 1478 Lørenskog, Norway

² Department of Clinical and Experimental Medicine University of Foggia Medical School Via L.Pinto 171122 Foggia, Italy

³CUHK-CAS GIBH Joint Research Laboratory on Stem Cell and Regenerative Medicine, School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong S.A.R., China

⁴Laboratory of Pre-clinical and Translational Research, IRCCS-CROB, Referral Cancer Center of Basilicata, 85028 Rionero in Vulture, Italy

⁵ Danish Aging Research Center, Department of Molecular Biology and Genetics, University of Aarhus, 8000 Aarhus C, Denmark

⁶Co-first authors

* Corresponding author: e.f.fang@medisin.uio.no or evandrofeifang@yahoo.com

Abstract

Aging is a natural and unavoidable part of life. However, aging is also the primary driver of the dominant human diseases, such as cardiovascular disease, cancer, and neurodegenerative diseases, including Alzheimer's disease. Unraveling the sophisticated molecular mechanisms of the human aging process may provide novel strategies to extend 'healthy aging' and the cure of human aging-related diseases. Werner syndrome (WS), is a heritable human premature aging disease caused by mutations in the gene encoding the Werner (WRN) DNA helicase. As a classical premature aging disease, etiological exploration of WS can shed light on the mechanisms of normal human aging and facilitate the development of interventional strategies to improve the healthspan. Here, we summarize the latest progress of the molecular understandings of WRN protein, highlight the advantages of using different WS model systems, including *C. elegans*, *Drosophila melanogaster* and induced pluripotent stem cell (iPSC) systems. Further studies on WS will propel drug development for WS patients, and possibly also for normal age-related diseases.

Keywords: aging; premature aging; Werner syndrome; NAD⁺; mitophagy; hallmarks of aging; DNA repair.

1. Introduction of Werner syndrome (WS)

1.1 Clinical Phenotypes

Werner syndrome (WS, OMIM#277700) is a rare autosomal recessive inherited disorder that displays clinical features indicative of accelerated aging (Bohr, 2005; Bohr et al., 2002; Oshima et al., 2017). WS is one of the few adult-onset accelerated aging syndromes, where the patients mostly develop normally until they reach adolescence (Oshima et al., 2017). The first clinical signs of WS appear as a lack of growth spurt in puberty, resulting in a relative short stature. In their 20s and early 30s, WS patients develop a prematurely aged appearance. This includes the development of a variety of age-related features including skin and muscle atrophies, loss of subcutaneous fat and graying and loss of hair. By the fourth decade of life bilateral cataracts, abnormal glucose and lipid metabolism, hypogonadism and bone deformity appear. Furthermore, WS is often accompanied by a series of common age-related diseases such as type II diabetes mellitus, osteoporosis, arteriosclerosis, and malignant neoplasms among others (Oshima et al., 2017; Yu et al., 1996). The most common causes of death for WS patients are cancer and myocardial infarction at a median age of 54 years (Goto et al., 2013; Huang et al., 2006). The median age of WS patients has been increasing during the last decades, likely due to improved medical care.

1.2 Etiology

Classical WS is caused by homozygous or compound heterozygous loss of function mutations in the *WRN* gene (Yu et al., 1996). The *WRN* locus is located on chromosome 8p12 and consists of 34 coding exons spanning a region of 140 kb (Yu et al., 1996). It encodes one of the five human RecQ-type DNA helicases, the WRN protein (Matsumoto et al., 1997; Oshima et al., 1996; Yu et al., 1996). The other four human RecQ-type DNA helicases are RECQL1, BLM, RECQL4 and RECQL5 (Croteau et al., 2014). Human WRN protein consists of 1432 amino acids with the size of 160 kDa. It is a multifunctional nuclear protein with a 3' to 5' exonuclease domain in its N-terminus (Huang et al., 2006), an ATP-dependent 3' to 5' helicase in its central region (Gray et al., 1997), a nuclear localization signal in its C-terminus (Matsumoto et al., 1997; Suzuki et al., 2001) and two additional consensus domains, namely the RecQ helicase conserved region (RQC) and the helicase, RNase D, C-terminal conserved region (HRDC). Fig. 1 gives a schematic illustration of the WRN protein.

The WRN helicase activity catalyzes unwinding of a broad spectrum of DNA substrates and intermediates, including intermediates of DNA replication such as complex G4-quadruplex structures, recombination and repair (Brosh and Bohr, 2002; Crabbe et al., 2004; Kamath-Loeb et al., 2012). The exonuclease activity of WRN degrades 3' recessed double stranded DNA, bubble, forked duplexes, Holliday junctions and the DNA in RNA-DNA duplexes (Huang et al., 2000; Shen and Loeb, 2000). WRN has been shown to have several interaction partners involved in DNA maintenance. These include proteins central for replication such as replication protein A (RPA), proliferating cell nuclear antigen (PCNA) and topoisomerase I (Lebel et al., 1999). WRN also interacts with multiple proteins involved in the DNA repair pathways including base excision repair (APE1, PARP-1, FEN1, Pol β) (Ahn et al., 2004), non-homologous end-joining (RAD51, RAD52, Mre11/Rad50/Nbs1) and homologous recombination (BRCA1, MRN, BLM) (reviewed in (Kusumoto et al., 2007)).

More than 80 different disease-causing mutations have been identified in *WRN* (Friedrich et al., 2010; Oshima et al., 2017; Uhrhammer et al., 2006). The majority of the mutations lead to premature stop codons or deletions, most of which result in truncations of nuclear localization signals at the C-termini and/or nonsense mediated decay of mutant mRNAs, therefore often referred to as null mutations (Oshima and Hisama, 2014; Oshima et al., 2017). In addition to mutations in the *WRN* locus, heterozygous mutations in the *LMNA* gene have also been associated with WS, and is referred to as atypical WS (AWS) (see review by (Chen et al., 2003) for more details). Currently, no clear correlation has been identified between the location of the mutation and the disease severity, thus further studies are needed to clarify this link.

Single nucleotide polymorphisms (SNPs) in the *WRN* gene have also been identified, and several polymorphisms have been associated with both longevity and disease risks though not all findings have been replicated in multiple cohorts (reviewed in (Lebel and Monnat, 2018)). The three different polymorphisms, L1074F, C1367R, and S1133A have been linked with longevity, though only the association between S1133A and longevity was significant (Castro et al., 2000; Kulminski and Culminskaya, 2013). Additionally, C1376R, S1133A and M387I have been associated with cardiovascular diseases (Castro et al., 2000; Kulminski and Culminskaya, 2013; Ye et al., 1997), while L1074F and C1367R have been connected with the risk of ischemic stroke (Sebastiani et al., 2013; Sebastiani et al., 2012). *WRN* gene SNPs have also been linked with dyslipidemia (V114I and S1133A) (Berube et al., 2013; Kulminski and Culminskaya, 2013) and diabetes (C1367R) (Hirai et al., 2005). Additionally, *WRN* gene SNPs have been associated with various types of cancers including bone and soft tissue sarcomas (C1367R), breast cancer (C1367R and V114I) and lung cancer (L1074F) (Gagne et al., 2016; Nakayama et al., 2008; Shen et al., 2006). In addition, C1367R has been associated with both bipolar disorder, schizophrenia and increased risk of Creutzfeldt-Jakob disease (Chen et al., 2013; Mead et al., 2012). Furthermore, SNPs in the 5' upstream region and 5' flanking areas of *WRN* have been associated with cognitive function (rs2251621, rs2725335, and rs2725338) despite mental impairment not being a typical feature of WS patients (Sild et al., 2006).

2. WS serves as a unique model to understand normal human aging

Aging is the primary driver of the dominant human diseases, such as cardiovascular disease and neurodegenerative diseases, while unraveling the underlying molecular mechanisms of the human aging process may provide novel strategies to extend 'healthy aging' and therapeutics for human aging-related diseases (Fang et al., 2014; Fang et al., 2016b). Due to a series of premature aging features WS has been considered as a great model for aging research (Bohr et al., 2002; Shamanna et al., 2017). When comparing characteristics of WS to the hallmarks of aging proposed by Lopez-Otin et al. (9 hallmarks of aging) and us (plus defective autophagy, including defective mitophagy) (Fang et al., 2017a; Lopez-Otin et al., 2013), WS has been linked with all the 10 hallmarks of aging: including epigenetic alterations (Maierhofer et al., 2017), telomere attrition (Crabbe et al., 2007; Wyllie et al., 2000), changes in DNA damage and repair (Cheng et al., 2006; Kusumoto et al., 2007; Opresko et al., 2004), deregulated nutrient sensing (Yasuda et al.,

2010; Yokote et al., 2004; Yokote and Saito, 2008), loss of proteostasis (Talaie et al., 2013; Zhu et al., 2015), altered cellular communication due to inflammation-induced elevated cytokine levels (Goto et al., 2015), cellular senescence (Faragher et al., 1993), stem cell exhaustion (Wu et al., 2018; Zhang et al., 2015b), and altered autophagy (Maity et al., 2014) and mitochondrial function (Cogger et al., 2014) (Fig. 2). In this review, we focus on the links between WS and some of these hallmarks, i.e., genomic stability, senescence, stem cells, mitochondrial function and autophagy.

2.1 WRN mutation in genomic stability

The two major characteristics of cells from WS patients are genomic instability and limited cell replicative lifespan (Oshima et al., 1995; Salk, 1985; Salk et al., 1985). WRN exhibits DNA-dependent ATPase, ATP dependent 3'→5' DNA helicase, single stranded DNA annealing and exonuclease activities. Through its various enzyme activities, WRN is able to resolve a variety of DNA substrates all representing intermediates in DNA replication and repair, and indeed WRN protein has been shown to be involved in multiple DNA transactions. WRN participates in both sub-pathways of double strand break repair (non-homologous end-joining and homologous recombination), base excision repair and telomere maintenance (Shamanna et al., 2016). In addition, WRN helicase unwinds and resolves complex DNA structures and intermediates including G-quadruplex structures (Kamath-Loeb et al., 2012). This activity includes the unwinding of G-quadruplex structures at the lagging telomere strand and defects in WRN has been shown to lead to replication fork stalling and degradation, hereby linking chromosome end maintenance to WS (Crabbe et al., 2004; Pichierri et al., 2001; Rodriguez-Lopez et al., 2002).

Furthermore, post-translational modifications of WRN modulate its enzymatic activity, thereby regulating its role in DNA maintenance processes (Kusumoto et al., 2007; Tadokoro et al., 2013). Additionally, oxidation of the WRN protein results in loss of its catalytic activities in addition to impairment of protein-protein interactions (Harrigan et al., 2007). Disease causing mutations such as the missense mutation c.1720G>A, p.Gly574Arg, has been shown to result in decreased helicase activity due to impaired ATP binding. Interestingly, WS patients with this mutation do not show the short stature normally seen in WS patients (Tadokoro et al., 2013). Thus, the short stature of WS patients may not be associated with impaired helicase activity but with the residual activities of WRN. Additionally, the WS patient carrying the G574R mutation in the study by Tadokoro *et al.*, shows nuclear WRN expression, whereas most WS patients carrying other WRN mutations do not. This could indicate that the G574R patient can sustain enough WRN activities/interactions to prevent growth failure, but still too little to prevent other WS related features (Tadokoro et al., 2013).

In addition to the above-mentioned functions, WRN also promotes telomere maintenance, and loss of WRN results in a rapid decline of telomere length, which has been linked to aging (Ishikawa et al., 2011; Opresko et al., 2004). Collectively, WRN plays a major role in DNA repair through its involvement in several DNA repair pathways.

2.2 WRN mutation in senescence

Cellular senescence refers to an irreversible growth arrest of primary eukaryotic cells and was first described for cells in culture (Hayflick, 1965). The process of senescence is thought to contribute to aging and aging-related degeneration (Lopez-Otin et al., 2013), where senescent cells are more common and might result in limited tissue renewal (Kong et al., 2011). Collectively, WS patient cells and WRN knock-down cells have been related with senescence and premature senescence. The clinical manifestations of WS, including a bird-like appearance, alopecia/gray hair, skin hyperpigmentation, hoarseness, diffuse arteriosclerosis, juvenile bilateral cataracts and osteoporosis, are all associated to premature senescence (Goto et al., 2013). Furthermore, primary skin fibroblasts from WS patients and WRN-deficient cells undergo early replicative senescence. Additionally, cells depleted for WRN show increased senescence-associated beta-galactosidase (SA- β -gal) staining, activation of the senescence-associated secretory phenotype (SASP) (Rodier and Campisi, 2011; Rodier et al., 2011) and accumulation of DNA damage foci (Lu et al., 2014). The premature senescence seen in WRN-deficient cells is related to the observed telomere shortening; overexpression of telomerase (hTERT) inhibits premature senescence at the same level as rescue with WRN protein and increases cellular lifespan (Crabbe et al., 2007; Grandori et al., 2003; Wyllie et al., 2000). WRN-null human embryonic stem cells (hESCs) differentiated to mesenchymal stem cells (MSCs) recapitulate features of premature cellular aging including changes of heterochromatin architectures in addition to altered epigenetic marks e.g. global loss of H3K9me3, all signs of premature senescence. Interestingly, MSCs from older individuals display decreased levels of WRN in addition to altered heterochromatin marks resembling the alterations seen in the WRN-null MSCs (Zhang et al., 2015b). Moreover, WS patients display a progressive increase in DNA methylation, considered as a prematurely increased epigenetic age (Maierhofer et al., 2017). Collectively, these studies suggest that loss of WRN promotes premature senescence, and that the role of WRN in the maintenance of various forms of DNA, including telomeres and heterochromatin might be important to aging.

2.3 WRN mutation in stem cell exhaustion

During normal aging the regenerative potential of stem cells is lost leading to an age-dependent stem cell exhaustion. The regulation of stem cells in WS is largely unknown, though recent studies indicate the importance of WRN in stem cell function. Normally, a balance between the quiescent and activated state of stem cells are kept to retain stem cell rejuvenation potential. Likely, WRN is central in the maintenance of this homeostasis. In addition to the above mentioned roles of WRN, links between WRN and the sirtuins (SIRT6) have previously been shown, indicating an important role of WRN in mitochondrial health (reviewed in (Chandel et al., 2016; Wrighton, 2015)). All of these factors, are known to be important contributors to the prevention of stem cell exhaustion (summarized in Fig. 3).

WRN-deficient hESCs and MSCs show phenotypes of accelerated aging including premature dysfunction such as decreased proliferative potential in addition to increased expression of p16^{INK4} and p21^{Waf1}, both markers of aging (Cheung et al., 2015; Shimamoto et al., 2014; Wu et al., 2018). Interestingly, WRN-depleted neuronal progenitor cells do not show signs of premature aging, neither do reprogrammed iPSCs,

likely due to an increased telomerase activity as a consequence of reprogramming (will be discussed in more detail below) (Cheung et al., 2014; Shimamoto et al., 2014). As explained above, WRN-null MSCs also display heterochromatin alterations linked to premature senescence, again suggesting WRN as an important contributor to chromatin regulation (Zhang et al., 2015b). ~~A role of WRN in chromatin regulation has also been suggested by a recent study indicating that treatment with vitamin C likely alleviates many features of premature aging seen in WRN-deficient MSCs via altered expression patterns of a series of genes involved in chromatin condensation, cell cycle regulation, DNA replication and DNA damage and repair (Li et al., 2016), though it needs further verification.~~ Thus, while evidence indicates a pivotal role of WRN in stem cell function (Fig. 3), more underlying molecular mechanisms are likely to be revealed.

2.4 WRN mutation in mitochondrial dysfunction and imbalanced autophagy

WS patient cells show mitochondrial dysfunction-related phenotypes. Thus, increased levels of reactive oxygen species (ROS) including both superoxide and hydrogen peroxide have been observed in WS patient cells. Furthermore, cells depleted for WRN, both mouse embryonic fibroblasts (MEFs) and human cancer cells including HeLa and breast cancer cells, show reduced levels of NADPH, a central provider of reducing equivalents for biosynthetic reactions and cellular protection against ROS. In addition, reduced levels of the antioxidant glutathione (GSH) and a metabolic shift resulting in altered mitochondrial respiration have been shown. All of the above mentioned consequences of WRN loss result in decreased cell proliferation (Li et al., 2014). Additionally, the WRN depleted cells showed increased levels of oxidative stress and also the oxidative DNA damage 8-oxoguanine (8oxodG) and the double strand break marker γ H2AX (Das et al., 2007). Interestingly, treatment with the antioxidant GSH normalized the stress level, mitochondrial function and proliferation of the WRN depleted cells to normal levels (Li et al., 2014), supporting an important role of ROS-induced oxidative stress and associated mitochondrial dysfunction in WS.

WRN has also been implicated in cellular metabolism and autophagy. Basal autophagy and the associated mTOR signaling have been found to be upregulated in WS cells (Saha et al., 2014; Talaei et al., 2013). While short-term rapamycin treatment increases the activation of autophagy, long-term treatment with rapamycin of WS cells results in improved growth rate, reduced accumulation of DNA damage and improved morphology of the nuclei. The autophagy markers LC3-II and p62 are also reduced after long-term rapamycin treatment (Saha et al., 2014), likely due to an increased clearance of dysfunctional and/or damaged cellular components consistent with enhanced degradation of autophagosomes (Saha et al., 2014). This effect of rapamycin treatment was found enhanced in WS cells compared to controls. In summary, there is a lack of comprehensive evaluation of mitochondrial phenotypes in WS and the role of WRN in autophagy is largely phenotypical. Further mechanistic studies of WRN in mitochondrial function and autophagy using isogenic cell lines are therefore necessary.

3. WS model systems

Most of the data available concerning the underlying mechanisms of WS are based on three model systems: fibroblasts and lymphoblastic cell lines derived from WS patients and two animal models, *Caenorhabditis elegans* (*C. elegans*) and *Drosophila*

melanogaster (*Drosophila*). Due to the well-known conservation of evolutionary pathways between species, both *C. elegans* and *Drosophila* can be used for drug development against human diseases. Despite mice being the generally most common animal model used for studying human diseases, it must be kept in mind that mice are more expensive to maintain in the laboratory, relatively long-lived and they require a very long process for genetic manipulation. In contrast, *C. elegans* and *Drosophila* models are cheaper to maintain, easy to manipulate and short-lived enabling large-scale lifespan and healthspan studies. One complication in studying the functions of WRN is its unique double DNA repair activities, where it functions both as an exonuclease and a helicase. Similar to human WRN, mouse WRN (mWRN) protein contains both helicase and exonuclease domains, making it difficult to disentangle the distinctive biological functions of the two domains. When modifying either one or both domains in mWRN, the mice lack a premature aging phenotype. This might be due the extended telomeres in mice compared to humans, since a premature aging phenotype appears in the telomerase-WRN double null mouse model (Chang et al., 2004), suggesting telomere shortening as an important component in WS. Therefore, the WS mouse models are not a preferable choice when studying WS. Unlike mammals, WRN activities are separated on different proteins in both flies and worms, enabling the separation of these activities and likely helping to understand its involvement at an organismal level.

3.1 WS *C. elegans*

In *C. elegans*, four RecQ family DNA helicases have been identified by comparing the genomic DNA sequences. These include the open reading frame T04A11.6, homologous with mammalian RecQL; HIM-6, corresponding to BLM; RCQ-5, equivalent to RecQ5; and the open reading frame F18C5.2 homologous with human WRN and therefore named WRN-1 in WormBase (<https://www.wormbase.org/>). No homolog of RecQ4 has been predicted in *C. elegans*. WRN-1 possesses only the helicase motif with a DEAH box which shares 43% identity in the amino acid sequence with that of the human WRN helicase domain. Moreover, the RQC (RecQ helicase conserved) domain and the HRDC domain share 27% identity with human WRN (Lee et al., 2004) (Fig. 1). In *C. elegans*, WRN-1 lacks the exonuclease domain, although the exonuclease domain of MUT-7 shares 29% identity with human WRN (Fig. 1) (Lee et al., 2004). Despite the homology, MUT-7 cannot be considered a functional homolog of WRN exonuclease, since it is involved in RNA interference and gene silencing (Ketting et al., 1999; Ryu and Koo, 2016), while the helicase activity has been shown to unwind various DNA structures (Hyun et al., 2008). WRN-1 and also mWRN are diffusively distributed in the nucleoplasm during interphase (Lee et al., 2004), where human WRN is mainly localized in the nucleolus (Marciniak et al., 1998; Suzuki et al., 2001). During S phase and as a response to DNA damage, human WRN translocates from nucleolus to specific foci in the nucleoplasm in connection with other DNA repair enzymes (Gray et al., 1998; Rodriguez-Lopez et al., 2003). How WRN-1 in *C. elegans* locates during cell cycle is currently unknown.

C. elegans wrn-1 mutant strains (including *wrn-1(gk99)*, *wrn-1(tm764)*, *wrn-1* (RNAi)) recapitulate several major phenotypes of WS patients, but also shows some distinctive features. The WRN-1 protein level has been shown to decrease with age in all tested tissues in adult worms (Lee et al., 2004). Notably, it has been shown that by silencing

wrn-1 using RNAi knockdown, worms have a shortened lifespan (from 13.6 of WT to 11.0 days in the *wrn-1*(RNAi) at 25°C). Additionally, *wrn-1*(RNAi) worms accumulate lipofuscin faster than wild type worms and dumpy body, whereas *wrn-1(gk99)*, *wrn-1(tm764)* and *wrn-1* (RNAi) worms show phenotypes including small body size, increased incidences of ruptured body, dumpy shape and growth arrest at larval stages (Lee et al., 2010; Lee et al., 2004), similar to clinical manifestations in human WS supporting the use of *wrn-1* mutant *C. elegans* as a model of human WS. *Wrn-1* (RNAi) worms also show an acceleration of larval growth and surprisingly early *wrn-1* (RNAi) embryos exhibit a shorter S-phase, which is in contrast to an elongated S-phase seen in WS patient cells (Lee et al., 2004). Indeed, mitotically proliferating germ cells in *wrn-1* (RNAi) worms show an ineffective checkpoint for DNA replication arrest even after hydroxyurea-induced stress. Moreover γ -radiation exacerbates the WS phenotype of *wrn-1* (RNAi) worms, while the faster growth rate is independent of ionization, suggesting that WRN-1 is involved in cellular responses to DNA damage (Lee et al., 2004). Additionally, WRN-1 has been proposed to be responsible for extensive end-resection. When WRN-1 is absent, it causes hyper-accumulation of RPA resulting in a failure of recruitment and phosphorylation of RAD-51, leaving the cells with an inefficient double strand break repair system (Ryu and Koo, 2016, 2017).

In general, *C. elegans* serves as a useful model for drug screening due to the highly conserved mechanisms, relative ease of use and short lifespan. Vitamin C treatment has been suggested to alter the expression of genes related to locomotion and anatomical structure, and to extend lifespan of the *wrn-1(gk99)* *C. elegans* strain, suggesting vitamin C as a potential drug against certain premature aging features in WS (Dallaire et al., 2012; Dallaire et al., 2014). Interestingly, vitamin C have also been suggested effective in the WS mice (Massip et al., 2010) as well as in a human mesenchymal stem cell model of WS (Li et al., 2016), though further studies are needed to confirm these findings. Combined, the current data suggest that *wrn-1* worms recapitulate some primary phenotypes of WS patients, and can serve as a powerful model for anti-WS drug screening.

3.2 *Drosophila* models of WS

In flies, an orthologue of the exonuclease domain of human WRN has been identified. Proteins encoded by the CG7670 and CG6744 loci have been identified as homologous to the human WRN exonuclease domain (Cox et al., 2007). CG7670 displays 34% homology, while CG6744 displays 33% homology with human WRN, respectively. CG6744 shares homology with the ATP-binding domain, the RQC region and C-terminal region of human WRN (Cox et al., 2007; Saunders et al., 2008). Additionally, CG6744 also shows 40% identity and 59% similarity with the exonuclease 3'-5' domain-like 2 protein (Fig. 1) (Cox et al., 2007). Thus, CG6744 has been assigned as the orthologue of human WRN, termed DmWRNexo (Cox et al., 2007). The helicase domain of human WRN has been found to share a high percentage amino acid identity and similarity (80%) with DmBLM encoded by the *mus309* locus (Fig. 1) (Cox et al., 2007; Kusano et al., 1999).

Despite the lack of a helicase domain, DmWRNexo reserves some important functions of human WRN and shows similar substrate specificity to human WRN exonuclease.

DmWRNexo shows 3' to 5' exonuclease activity on both single stranded and 5' overhang duplex templates but not on blunt ends dependent on divalent cations (Mg^{2+}) (Boubriak et al., 2009; Mason et al., 2013), similar to human WRN (Huang et al., 1998; Machwe et al., 2000; Machwe et al., 2011). Additionally, DmWRNexo is involved in restarting stalled replication forks, and mutations in its locus lead to hyper-recombination and camptothecin hypersensitivity, as already shown in human WS cells (Machwe et al., 2011; Saunders et al., 2008). Studies have also demonstrated how DmWRNexo plays a key role already in the early embryogenesis. Indeed, fly embryos carrying a mutation in the exonuclease domain of DmWRNexo, undergo slower replication. This causes replicative fork arrest leading to accumulation of DNA damage, resulting in improper nuclear division and embryonic development (Bolterstein et al., 2014). Similar features have already been observed in WS and WRN-depleted human cells (Opresko et al., 2007; Pichierri et al., 2001; Szekely et al., 2005). Combined the great similarity between hWRN and DmWRNexo on both sequence and activity levels extends the usability of *Drosophila* as a powerful model of human WS.

3.3 WS Mice

mWRN shares ~70% amino acid identity with that of the human WRN protein and it exhibits both helicase and exonuclease activity (Huang et al., 2000) (Fig. 1). There are at least three WS mouse models available. These models include mice lacking the entire WRN protein (*Wrn null* or *Wrn*^{-/-} mice), mice carrying a deletion in the helicase domain (*Wrn* ^{Δ hel/ Δ hel} mice) and transgenic mice expressing human WRN with a dominant-negative mutation (K577M-WRN) (Aumailley et al., 2015; Huang et al., 2000; Lebel et al., 2003; Massip et al., 2006). *Wrn*^{-/-} and *Wrn* ^{Δ hel/ Δ hel} mice have been well-characterized. The *Wrn*^{-/-} mice show increased DNA damage sensitivity, but surprisingly they do not exhibit accelerated aging features, possibly due to the long telomeres compared to humans (Chang et al., 2004). Indeed, Chang et al., showed that combined telomere dysfunction and *WRN* depletion in mice manifests features seen in human WS such as replicative senescence leading to premature aging and tumorigenesis (Chang, 2005). Thus the *Terc*^{-/-} *Wrn*^{-/-} mice recapitulate many of the phenotypes of human WS, showing a key role of telomere maintenance in WS and the aging process (Chang et al., 2004).

Conversely, the *Wrn* ^{Δ hel/ Δ hel} mice recapitulate most of the WS phenotypes, such as abnormal hyaluronic acid secretion, higher systemic ROS levels, dyslipidemia, heart failure, increased genomic instability and different types of cancers (Lebel et al., 2001; Lebel and Leder, 1998; Massip et al., 2006). Moreover, these mice have a shorter mean lifespan (reduced 10-15% when compared to wild type). As also seen in human cells and *C. elegans* models, vitamin C increases the lifespan and healthspan of *Wrn* ^{Δ hel/ Δ hel} mice (Lebel et al., 2003; Massip et al., 2010). Mislocalization of WRN mutant protein to organelles including peroxisomes, endoplasmic reticulum and autophagosomes, rather than to the nucleus, likely is responsible for the premature aging phenotypes (Aumailley et al., 2015).

WRN K577M mice show abolished ATPase and helicase activity but a retained exonuclease activity. In addition, tail fibroblasts from K577M-WRN transgenic mice, exhibit hypersensitivity to the genotoxic agent 4-nitroquinoline-1-oxide (4NQO) and

slower proliferative capacity, even though these mice do not show any pathophysiological feature linked to WS (Wang et al., 2000).

3.4 Human WS iPSCs

The availability of WS iPSCs has provided a new and powerful approach to study WS, through the provision of isogenic background and the differentiation of any types of cells of interest. Generation of WS iPSCs allows researchers to unveil pathophysiological mechanisms and also test the newest pharmacological treatments in a human context. Currently, most of the data on WS are limited to patient-derived fibroblasts and lymphocytes. *WRN*^{-/-} hESCs have been established and differentiated to MSCs as explained earlier. The *WRN*^{-/-} MSCs exhibited features of premature cellular aging, including premature loss of proliferative potential and epigenetic and chromatin structure alterations (Zhang et al., 2015a). Although these cells provide a reasonable model of WS, a human iPSCs line would allow the *in vitro* reconstruction of the disease. Currently, the generation of human iPSCs from WS patients is limited to three cases, describing a successful generation of iPSCs through the introduction of several pluripotency genes resulting in induction of the gene encoding human telomerase reverse transcriptase (hTERT) (Cheung et al., 2014; Shimamoto et al., 2014; Wang et al., 2018). When fully reprogrammed to iPSCs, the cells completely lost the WS related phenotype in addition to restored telomerase levels, opposite to the original WS patient fibroblasts. Additionally, the karyotype of the iPSCs remained stable over multiple passages (Fig. 3) (Shimamoto et al., 2015). A strong interplay between WRN and telomere maintenance has previously been observed, confirming the importance of telomere maintenance. Induction of hTERT in the reprogramming process from WS patient fibroblasts to iPSCs recovers the telomerase activity, which causes elongation of telomeres and hereby an extended cellular lifespan (Shimamoto et al., 2015). These data demonstrate that the premature senescence observed in WS fibroblasts likely is due to an insufficient activity of telomerase, and that expression of hTERT recovers the phenotype when generating iPSCs (Cheung et al., 2014; Shimamoto et al., 2014). Results from these iPSC studies suggest that WS is a stem cell dysfunction-associated disease. However, since WS is a segmental progeroid syndrome, features associated with aging (such as dementia) do not completely overlap with WS. Despite the apparent aging phenotypes observed in mesenchymal stem/progenitor cells and fibroblasts, lack of premature senescence is observed in neural stem cells, keratinocytes (Ibrahim et al., 2016) and endothelial cells (Wu et al., 2018). It raises the question whether *WRN* mutation equally affects all the lineages and tissues. Apart from senescence, other hallmarks of aging have not been systematically examined in different adult stem/progenitor cells (Fig. 3). Thus, more studies are needed to differentiate the effects of *WRN* depletion in various iPSC-derived cell types

4. Outstanding questions and future perspectives

The premature aging features of WS can to a large extent be connected to several of the hallmarks of normal aging including telomere attrition, genomic instability and altered cellular communication, among others (Fig. 2). Extensive studies on the mechanisms behind WS are warranted. First, how *WRN* regulates metabolic homeostasis and whether this is by controlling the mitochondrial quality and function, should be studied. Second, it

would be interesting to look for potential changes in the DNA damage-induced signaling from the nucleus to mitochondria (NM signaling) in WS. Accordingly, recent studies suggest mitophagy and NAD⁺ depletion to be central in the aging process and NAD⁺ precursor supplementation has shown tremendous positive effects on premature aging disorders including Cockayne syndrome, Xeroderma pigmentosum group A, and Ataxia telangiectasia (Fang et al., 2016a; Fang et al., 2017b; Fang et al., 2014; Fang et al., 2016b; Scheibye-Knudsen et al., 2014). This drives us to speculate whether mitophagy and/or NAD⁺ metabolism also affect WS, and this is indeed the case (Fang EF et al., unpublished data). Third, it has so far been challenging to divide the roles of the exonuclease and helicase domain of WRN in the features of WS. The more recent worm and fly models of WS, might be able to help elucidate and distinguish the roles of the domains in the human syndrome. Additionally, more extensive research of WS stem cells may also help to clarify the mechanistic failures during human WS.

In summary, WS is a classical premature aging disease with mechanisms still elusive. Combining different WS systems, including worms, flies, mice and human stem cells, may dramatically facilitate our research on WS as well as to broaden our understanding of the multi-faceted roles of WRN in the mechanism of normal aging. Additionally, cross-species studies of WS will propel the development of efficient drugs for this currently incurable disease.

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Figures

Fig. 1. Comparison of conserved regions of WRN in WS humans and animal models. Amino acid identities with human WRN are indicated below WS model proteins. R = acidic repeats; RQC = RecQ conserved domain; HRDC = helicase Rnase D conserved domain; NLS = nuclear localization signal. The numbers just below the illustration of the various domains refer to the amino acids in the protein sequence. The domains are drawn based on *H. sapiens*: UniProtKB - Q14191 and (Lebel and Monnat, 2018); *M. musculus*: UniProtKB - O09053 and (Chen and Oshima, 2002); *C. elegans* wrn-1: UniProtKB - Q19546 and (Lee et al., 2004; Ryu and Koo, 2016); *C. elegans* mut-7: UniProtKB - P34607 and (Lee et al., 2004); *D. melanogaster* WRN: UniProtKB - Q9VE86 and (Cox et al., 2007; Saunders et al., 2008); *D. melanogaster* BLM encoded by mus309 locus: UniProtKB - Q9VGI8, (Cox et al., 2007; Kusano et al., 1999). RecQ5 and RecQ4 also show high similarity to human WRN, but lower than that of DmBLM (Cox et al., 2007).

Fig. 2. WS and its relation to the 10 hallmarks of aging. WS can be related to many of the hallmarks of aging proposed by us and others (Fang et al., 2017a; Lopez-Otin et al., 2013), indicated outside the circle. Within the colored areas, clinical characteristics, metabolites and proteins involved in the links between WS and the hallmarks of aging are shown. While the linkages between WRN dysfunction and many of the hallmarks of aging are extensively studied, the linkages between WRN dysfunction and stem cell exhaustion, imbalanced autophagy, and mitochondrial dysfunction, respectively (boxed) are largely elusive and need further investigation. For detailed information, please see the text and references found here. Image credit of the WS patient (48 years old): William and Wilkens publishing Inc.

Fig. 3. WRN and its effect on stem cells. (A) Representative images of fibroblasts, fibroblast-induced pluripotent stem cells (iPSCs), and further iPSC-differentiated mesenchymal stem cells (MSCs) from both a WS patient and a matched healthy control. WS primary fibroblasts and MSCs show much higher levels of senescence (blue colored β -Gal staining) compared with respective healthy control cells. Scale bar, 100 μ m. **(B)** A list of features associated with the reprogramming of WS fibroblasts to iPSCs. **(C)** Roles of WRN protein in stem cell function in normal MSCs (blue panel) and the putative consequences of loss of WRN in WS MSCs (red panel). The dashed lines indicate the roles of mitochondrial sirtuins (SIRT3, 4, 5) in the regulation of mitochondrial homeostasis through mitophagy, which has been established in normal human cells, but needs to be

verified in MSCs. In addition, WRN dysfunction inhibits NAD⁺/SIRT1 activity (Fang EF et al., unpublished data). For detailed information, please see the text and cited references.

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